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Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle[1]

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ABSTRACT: Cattle are a natural reservoir of the food-borne pathogen *Escherichia coli* O157:H7. Therefore, strategies that reduce *E. coli* O157:H7 prior to slaughter will reduce human exposures to this virulent pathogen. When bacteria that can anaerobically respire on nitrate (e.g., *E. coli*) are exposed to chlorate, they die because the intracellular enzyme nitrate reductase converts nitrate to nitrite, but also co-metabolically reduces chlorate to cytotoxic chlorite. Because chlorate is bactericidal only against nitrate reductase-positive bacteria, it has been suggested that chlorate supplementation be used as a strategy to reduce *E. coli* O157:H7 populations in cattle prior to harvest. Cattle (n = 8) were fed a feedlot-style high-grain diet experimentally infected with three strains of *E. coli* O157:H7. Cattle were given access to drinking water supplemented with 2.5 mM KNO$_3$ and 100 mM NaCl (controls; n = 4) or 2.5 mM KNO$_3$ and 100 mM NaClO$_3$ (chlorate-treated; n = 4). Sodium chlorate treatment for 24 h reduced the population of all *E. coli* O157:H7 strains approximately two logs (10^4 to 10^2) in the rumen and three logs (10^6 to 10^3) in the feces. Chlorate treatment reduced total coliforms and generic *E. coli* from 10^6 to 10^4 in the rumen and by two logs throughout the rest of the gastrointestinal tract (ileum, cecum, colon, and rectum). Chlorate treatment reduced *E. coli* O157:H7 counts throughout the intestinal tract but did not alter total culturable anaerobic bacterial counts or the ruminal fermentation pattern. Therefore, it appears that chlorate supplementation is a viable potential strategy to reduce *E. coli* O157:H7 populations in cattle prior to harvest.

Key Words: *Escherichia coli* O157:H7, Chlorate, Cattle

Introduction

Escherichia coli is a common member of the gastrointestinal microflora of animals and humans (Drasar, 1974), but the strain O157:H7 has become notorious following repeated outbreaks of hemorrhagic colitis in humans that have claimed several lives (Mead, 2000). Cattle are a natural reservoir of this food-borne pathogenic bacterium (Hancock et al., 1998; Rasmussen et al., 1993); and recent evidence has shown that approximately 28% of all cattle in the United States are infected with *E. coli* O157:H7 (Elder et al., 2000). The United States has one of the safest food supplies in the history of the world, yet approximately 73,000 people are sickened each year by *E. coli* O157:H7 (Mead et al., 1999). Several human outbreaks have been linked to improperly handled ground beef; therefore, *E. coli* O157:H7 has become closely associated in the public consciousness with bovine-derived products.

Some intestinal bacteria are able to anaerobically reduce nitrate to nitrite (e.g., *E. coli*) via the intracellular enzyme nitrate reductase (Alaboudi, 1982). Nitrate reductase also co-metabolically reduces chlorate to form the cytotoxic end-product chlorite (Stewart, 1988). Thus, when *E. coli* O157:H7 is exposed to chlorate, it dies (Anderson et al., 2000a; Callaway et al., 2001). Chlorate addition to in vitro cattle ruminal fluid fermentations significantly reduced *E. coli* O157:H7 populations (Anderson et al., 2000a). Preliminary in vivo studies have indicated that intraruminal addition of chlorate in cattle reduced fecal *E. coli* populations (Anderson et al., 2000b). Based on these results, it was hypothesized that cattle treated with chlorate prior to slaughter would contain lower *E. coli* O157:H7 populations than untreated cattle. The present study was designed to examine the effect of one-time sodium chlorate administration via drinking water on intestinal popula-

Table 1. Composition of feedlot diet fed to cattle before inoculation with *E. coli* O157:H7 strains and during chlorate treatment (on DMI basis)

Item	%
Cracked corn	74.4
Soybean meal	9.2
Urea	0.7
Trace mineral salt	0.4
Coastal Bermuda hay	15.3

tions of *E. coli* O157:H7, as well as generic *E. coli*, total coliforms, and total culturable anaerobic bacteria.

Materials and Methods

Cattle Diets and Experimental Design

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC protocol 00-002). Four ruminally fistulated and four nonfistulated , nonlactating Holstein cows (average 900 kg BW) were adapted to a high-grain diet (Table 1) in a stepwise fashion. Cows were fed according to NRC recommendations (NRC, 2000) and were allowed ad libitum access to water. Two ruminally fistulated and two nonfistulated animals were randomly assigned to each treatment (control or chlorate-treated). Cattle (n = 8) were housed in environmentally controlled facilities and were screened for the presence of *E. coli* capable of growth on antibiotic-supplemented agar prior to experimental inoculation with *E. coli* O157:H7 strains.

Bacterial Cultures

Escherichia coli O157:H7 strain 933 (ATCC 43895), strain 6058 (graciously provided by Dan Rice, Field Disease Isolation Unit, Washington State Univ., Pullman), and strain 86-24 (graciously provided by Francisco Diez-Gonzalez, Dept. of Food Sci. and Nutr., Univ. of Minnesota, St. Paul) were cultivated in anoxic Tryptic Soy Broth (**TSB**) medium at 37°C. Strains 933 and 6058 were isolated from human victims of hemorrhagic colitis outbreaks caused by ingestion of improperly cooked ground beef. Strain 933 was resistant to novobiocin and nalidixic acid (20 and 25 µg/mL, respectively), strain 6058 was resistant to rifampicin (25 µg/mL), and strain 86-24 was resistant to streptomycin (100 µg/mL).

Overnight cultures (1,000 mL each) of all three strains of *E. coli* O157:H7 were harvested by centrifugation (7,500 × *g*, 24°C, 10 min) and the cell pellets of all three strains were mixed and collectively resuspended in TSB medium (150 mL). Each cow was inoculated with *E. coli* O157:H7 (1×10^{10} cfu of strain 933, 2×10^{10} cfu of strain 6058, and 3×10^{10} CFU of strain 86-24) through the ruminal fistula or via injection through the paralumbar fossa at –48 h (Figure 1). Ruminal fluid samples were collected via ruminal cannula (fistulated

cows only) and were strained through sterilized nylon paint strainers (0.1 mm mesh size) and fecal samples were collected via rectal grab (all cows) every 12 h following inoculation. Fecal and ruminal populations of each O157:H7 strain, as well as generic *E. coli*, total coliforms, and total culturable anaerobes were enumerated as described below.

Chlorate Treatment

Feed and water were withdrawn from cattle for 24 h to stimulate water intake (Figure 1). Cattle were then given access to full feed and ad libitum access to drinking water supplemented with either 2.5 m*M* KNO$_3$ and 100 m*M* NaCl (controls) or 2.5 m*M* KNO$_3$ and 100 m*M* NaClO$_3$ (chlorate-treated). Potassium nitrate was included in treatments in order to stimulate induction of nitrate reductase activity among the intestinal microflora. Cattle received control or chlorate-supplemented drinking water treatments for 24 h prior to harvest.
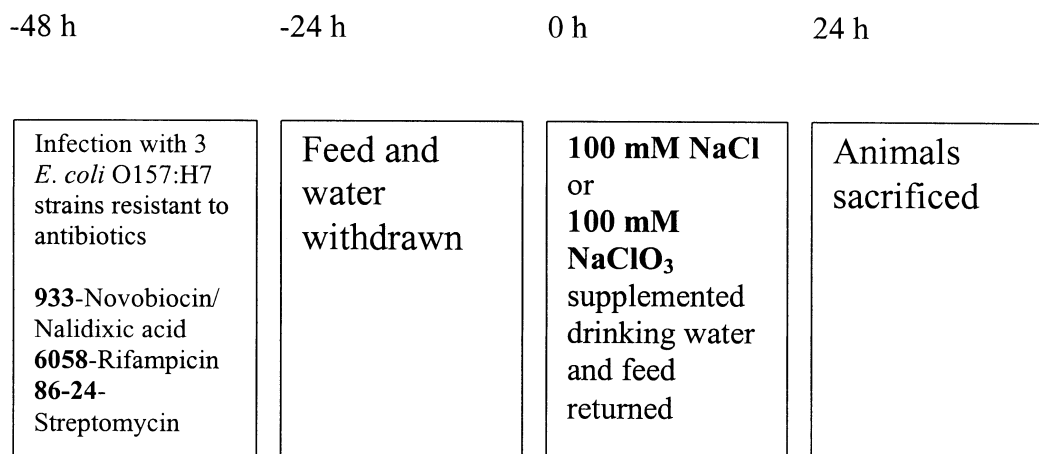
Hide Swabs and Gastrointestinal Sample Collection

Cattle were humanely euthanatized and exsanguinated. Hide swabs were performed on a 15.2- × 15.2-cm area of the hock and ventral midline of the hanging carcass using sterile gauze pads saturated with sterile PBS (pH 7.0) and sterilized metal templates. Gauze pads were immediately placed in sterile bags for transport to the laboratory. Sterile PBS was added to the bags containing hide swipe pads to dilute material from the hide 10-fold, and bags and swipes were mechanically massaged (via Stomacher) for 1 min to thoroughly mix each sample. Fluid from this initial dilution was used for subsequent dilutions and enrichments of hide swipes.

Intestinal contents and epithelial tissues from the rumen, ileum, cecum, colon, and rectum were aseptically collected upon necropsy as grab samples. Samples were diluted as described below for quantitative enumerations. Sample aliquots and epithelial tissues were added to TSB for overnight qualitative enrichment of *E. coli* O157:H7 strains. Overnight enrichments were plated as described below. Samples of ruminal, cecal, and rectal contents were immediately transferred to sealed tubes containing anoxic reinforced clostridial agar (described below) for determination of total culturable anaerobic bacteria as described below.

Bacterial Enumeration

Ruminal, intestinal, and fecal contents were serially diluted (10-fold increments) in sterile PBS. Dilutions were plated on MacConkey's agar (for total coliforms), MacConkey's agar supplemented with novobiocin (20 µg/mL) and nalidixic acid (25 µg/mL), rifampicin (25 µg/mL), or streptomycin (100 µg/mL) (for inoculated *E. coli* O157:H7 strains 933, 6058, and 86-24, respectively), and M-Endo agar LES (for enumerating total

-48 h   -24 h   0 h   24 h

Infection with 3 *E. coli* O157:H7 strains resistant to antibiotics **933**-Novobiocin/ Nalidixic acid **6058**-Rifampicin **86-24**-Streptomycin	Feed and water withdrawn	**100 mM NaCl** or **100 mM NaClO₃** supplemented drinking water and feed returned	Animals sacrificed

Figure 1. Experimental design. Cattle (n = 8) were inoculated with *E. coli* O157:H7 strains at −48 h. Feed and water was withdrawn at −24 h and control or chlorate supplementation begun at 0 h. Animals were killed at 24 h.

E. coli) and incubated overnight at 37°C. Colonies that grew on agar plates after a 24-h incubation were directly counted (quantitative enumeration). To qualitatively confirm the presence of inoculated *E. coli* O157:H7 strains, intestinal contents and epithelial tissue samples were incubated overnight in TSB at 37°C and were streaked on all three antibiotic-supplemented MacConkey's agars. Plates that contained colonies after a 24-h incubation were classified as positive for each *E. coli* O157:H7 strain.

Most probable number (**MPN**) estimates of total culturable anaerobic bacteria from ruminal, cecal, and rectal fluid were determined by serially diluting anaerobic samples harvested at necropsy and performing a triplicate three-tube MPN test using anoxic reinforced clostridial agar supplemented with 1.67 mM xylose, 0.73 mM cellobiose, and 40% filter-sterilized ruminal fluid. Tubes were incubated at 37°C, turbidity after 7 d was indicative of growth in each dilution, and MPN values were estimated using standardized calculations (AOAC, 1980). Intestinal contents were analyzed for pH and VFA concentrations as previously described (Corrier et al., 1990).

Reagents and Supplies

Unless otherwise noted, all media and agar were from Difco Laboratories, Detroit, MI. Reagents and antibiotics were obtained from Sigma Chemical Co., St. Louis, MO.

Statistics

Data were compared via Student's *t*-test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Results

Prior to inoculation with *E. coli* O157:H7 strains, cattle did not contain ruminal or fecal *E. coli* popula-

tions capable of growth on agar containing concentrations of antibiotics used to differentiate each inoculated *E. coli* O157:H7 strain (data not shown). Following inoculation with *E. coli* O157:H7 strains (12 h), populations of each strain of *E. coli* O157:H7 ranged from 10^5 to 10^7 cfu/g of feces and 10^5 to 10^6 cfu/mL of ruminal fluid (Figures 2a and b). Populations of *E. coli* O157:H7 remained stable in ruminal fluid and feces until chlorate treatment commenced (at 0 h). Fecal populations of all three inoculated strains of *E. coli* O157:H7 in control cattle remained constant (10^6 cfu/mL) throughout the study, but chlorate treatment reduced fecal populations ($P < 0.05$) more than 100-fold (Figure 2a). When water (supplemented with 100 mM NaCl or 100 mM NaClO₃) was returned to the cattle after a 24-h withdrawal period, cattle drank copiously; water intake between controls and chlorate-treated groups were not different (43.8 L vs 44.9 L per day per animal). Water intake diluted ruminal contents and caused a decrease ($P < 0.05$) in ruminal concentrations of all three inoculated *E. coli* O157:H7 strains in both treatment groups (Figure 2b). However, after 24 h of chlorate treatment initiation, populations of each *E. coli* O157:H7 strain in control animals were 10^4 to 10^5 cfu/mL, but ruminal populations were reduced ($P < 0.05$) in chlorate-treated cattle by more than 100-fold (Figure 2b).

Throughout the gastrointestinal tract, chlorate treatment reduced total coliform, *E. coli*, and *E. coli* O157:H7 populations (Figures 3a and b). Total coliforms and generic *E. coli* were reduced approximately 100-fold in the rumen ($P < 0.05$) and were reduced approximately 1,000-fold throughout the lower intestinal tract ($P < 0.05$) (Figure 3a). Inoculated *E. coli* O157:H7 strains comprised a small proportion of the total *E. coli* population of the lower intestinal tract (always less than 10%). Chlorate treatment decreased populations ($P < 0.05$) of all three strains of inoculated *E. coli* O157:H7 by at least 100-fold ($P < 0.05$) throughout the gastrointestinal tract (Figure 3b). All intestinal samples and intestinal epithelial tissues that underwent qualitative enrich-
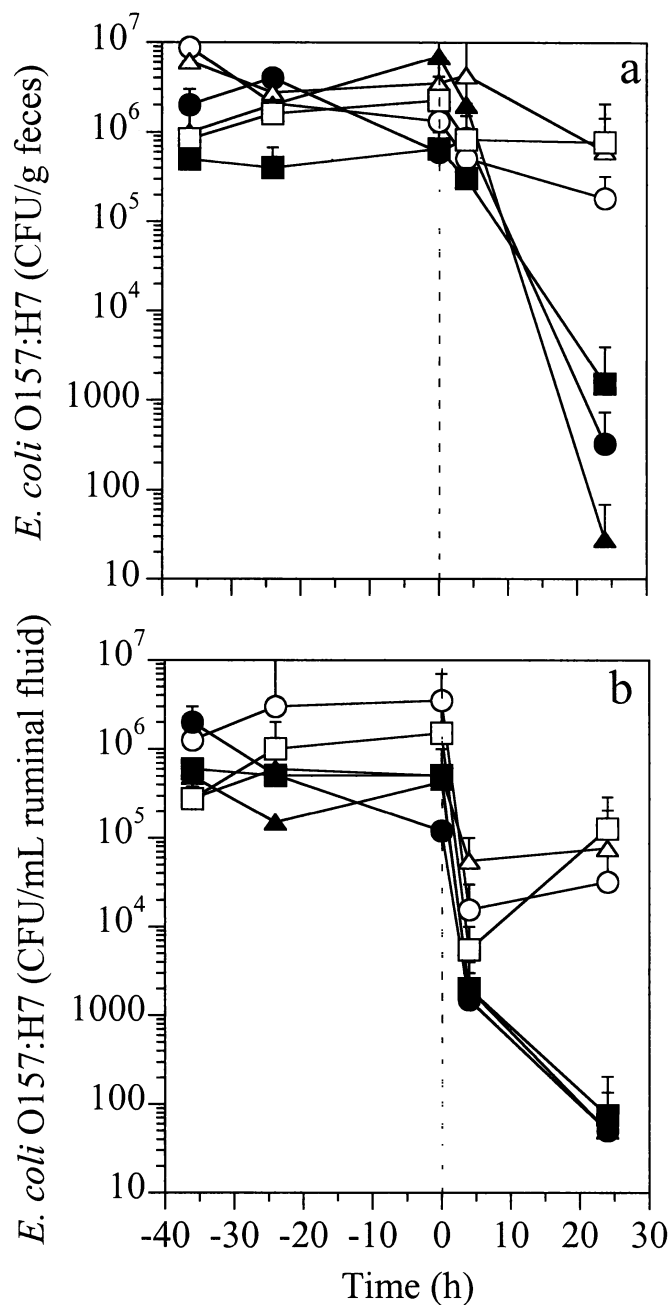
Figure 2. *Escherichia coli* O157:H7 CFU/g in feces (a) and cfu/mL ruminal fluid (b) from cattle. Ruminal fluid samples represent n = 2 animals/treatment and fecal samples were with n = 4 animals/treatment. Initiation of chlorate supplementation is indicated by the vertical dashed line. Strain 933 is indicated by (○, ●), strain 6058 by (□, ■), and strain 86-24 by (△, ▲). *E. coli* O157:H7 populations from cattle treated with 100 m*M* sodium chloride are indicated by open symbols, and populations from cattle that received 100 m*M* sodium chlorate are indicated by closed symbols. Standard deviations are indicated by error bars.
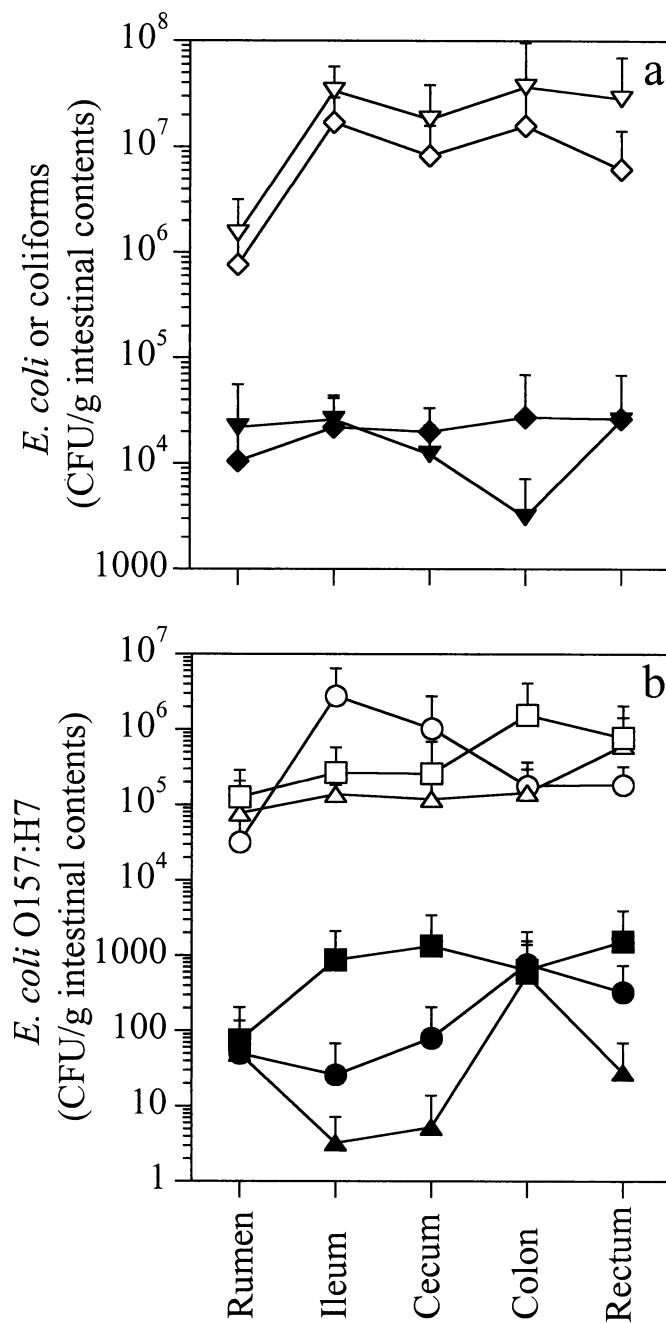
Figure 3. Total coliform (◇, ◆) and *Escherichia coli* (▽, ▼) populations in gastrointestinal contents are shown in (a) and *Escherichia coli* O157:H7 strains in gastrointestinal contents are shown in (b). Strain 933 is indicated by (○, ●), strain 6058 by (□, ■), and strain 86-24 by (△, ▲). *E. coli*, coliform and *E. coli* O157:H7 populations from cattle treated with 100 m*M* sodium chloride (n = 4) are indicated by open symbols, and populations from cattle that received 100 m*M* sodium chlorate (n = 4) are indicated by closed symbols. Standard deviations are indicated by error bars.
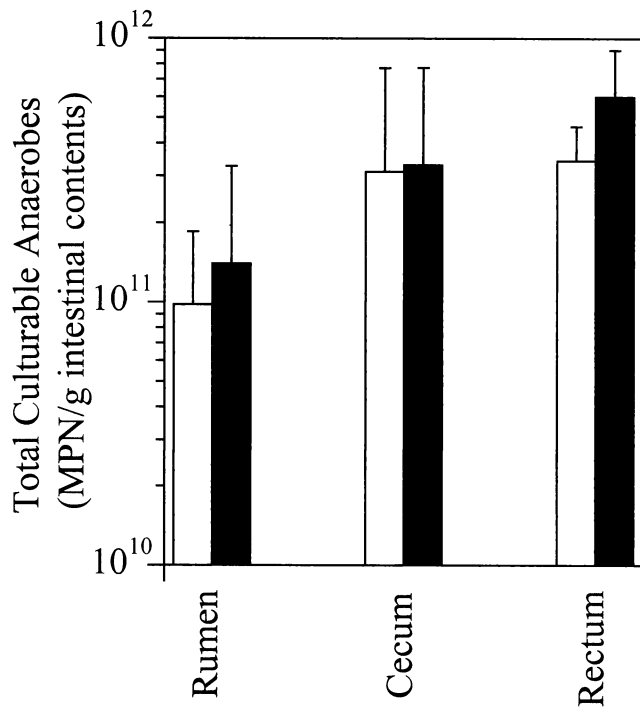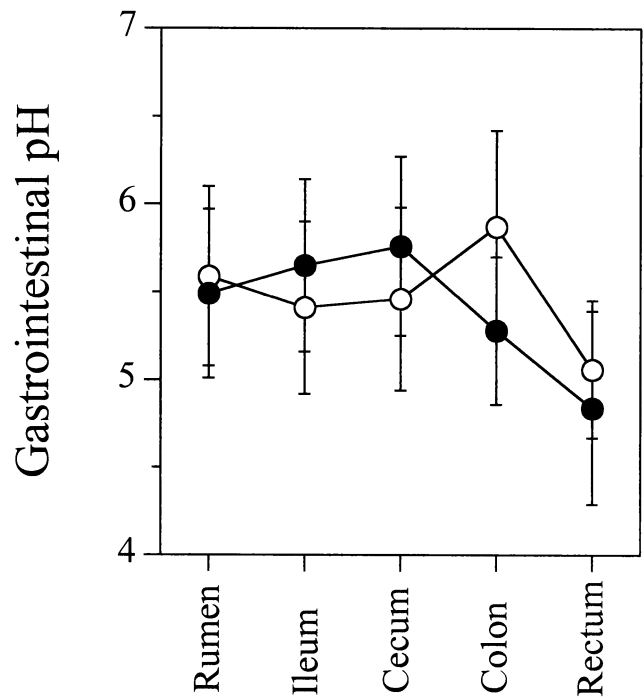
Figure 4. Total anaerobic bacterial populations (MPN/ mL) in gastrointestinal contents. Populations from cattle treated with 100 m*M* sodium chloride (n = 4) are indicated by open bars, and populations from cattle that received 100 m*M* sodium chlorate (n = 4) are indicated by filled bars. Standard deviations are indicated by error bars.

Figure 5. Gastrointestinal pH of cattle treated with 100 m*M* sodium chloride (n = 4) (open symbols) or 100 m*M* sodium chlorate (n = 4) (filled symbols). Error bars depict standard deviations.

ment contained all three strains of *E. coli* O157:H7 (data not shown).

Total culturable anaerobic bacterial populations of the rumen, cecum, and colon were not affected by chlorate treatment (Figure 4). Chlorate treatment did not significantly affect gastrointestinal pH (Figure 5). The fermentation profile throughout the gastrointestinal tract was not significantly affected by chlorate treatment, as indicated by total VFA concentration and the acetate:propionate ratio (Figures 6a and b). Hide swabs from the ventral midline and hock contained < 10^2 cfu/ cm^2 of each *E. coli* O157:H7 strain (data not shown). Because *E. coli* O157:H7 populations were so low, chlorate treatment did not affect populations of O157:H7 strains, coliforms, or generic *E. coli* recovered from hide swabs of the ventral midline or the hock.

Discussion

Cattle are commonly colonized by *E. coli* O157:H7 but cannot be visually identified as "sick" because they lack toxin receptors and, therefore, do not suffer from hemorrhagic colitis (Cray and Moon, 1995; Pruimboom-Brees et al., 2000). Research performed during the past decade indicated that the prevalence of *E. coli* O157:H7 in cattle was only 1 to 3% (Hancock et al., 1998). However, more recent studies using molecular techniques

have indicated that the prevalence in cattle is much greater (Bielaszewska et al., 2000; Elder et al., 2000). Up to 30% of cattle have been shown to be carriers of *E. coli* O157:H7 (Elder et al., 2000), but the incidence of fecal shedding varies with season and is highest during summer months (Hancock et al., 1998).

Approximately 75% of all *E. coli* O157:H7 outbreaks in humans have been linked to bovine-derived products (USDA:APHIS, 1997), and this has led to the description of *E. coli* O157:H7 as "hamburger disease" or "feedlot disease" (Martens, 2000). Processing plants successfully reduce *E. coli* O157:H7 contamination in the abattoir (Elder et al., 2000); however, illnesses and massive ground beef recalls still frequently occur. Food safety research has primarily focused on post-harvest intervention methods; thus, strategies designed to reduce pathogenic bacterial populations in the animal prior to entry to the food chain could further reduce the numbers of resulting human illnesses.

Sodium chlorate kills bacteria that can anaerobically respire on nitrate via a dissimilatory nitrate reductase (e.g., *E. coli*, *Salmonella*, and *Selenomonas*) but does not kill bacteria lacking nitrate reductase (e.g., strict anaerobes) (Ingledew and Poole, 1984; Stewart, 1988). Sodium chlorate treatment has been shown to eliminate both *E. coli* O157:H7 and *Salmonella typhimurium* DT104 in ruminal fluid incubations (Anderson et al., 2000a), *E. coli* O157:H7 in bovine fecal fluid incubations (Callaway et al., 2001), and *Salmonella typhimurium* and *E. coli* O157:H7 in swine (Anderson et al., 2001).
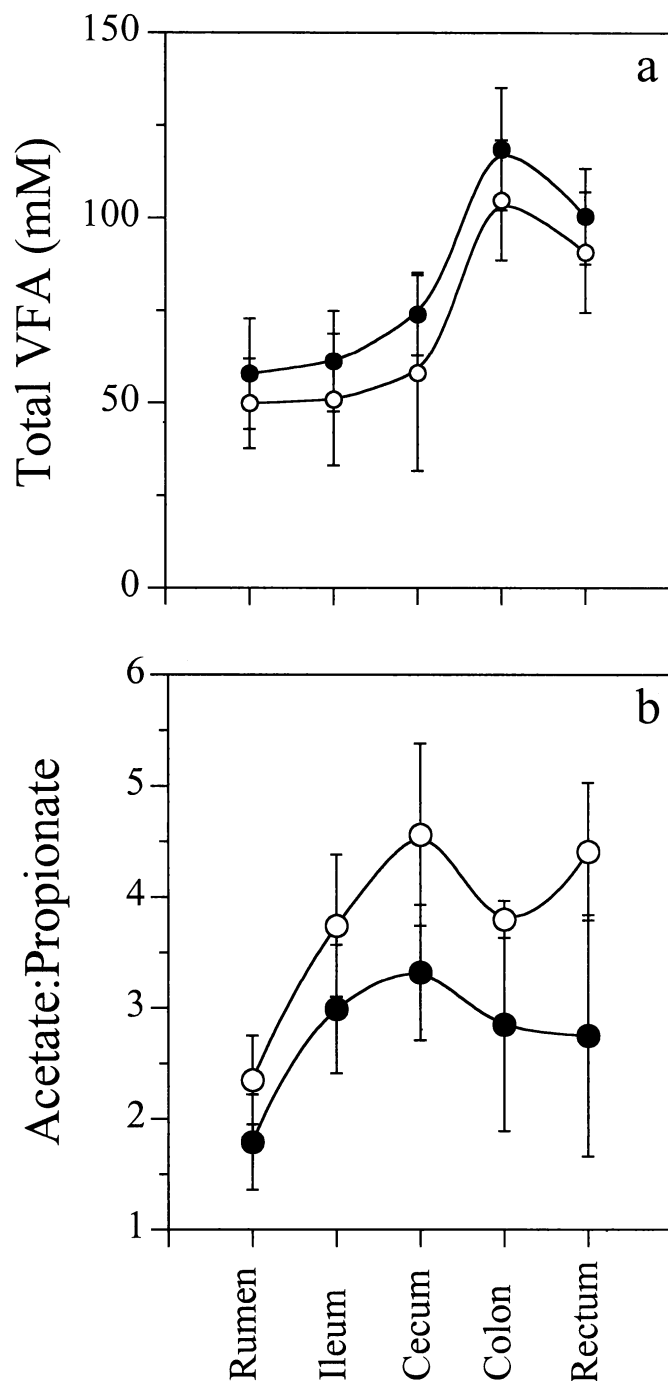
Figure 6. Gastrointestinal total VFA concentrations are shown in (a) and the acetate:propionate ratio is shown in (b). Samples from cattle treated with 100 m*M* sodium chloride (n = 4) are indicated by open symbols, and samples from cattle that received 100 m*M* sodium chlorate (n = 4) are indicated by filled symbols. Standard deviations are indicated by error bars.

Further studies indicated that the intraruminal addition of sodium chlorate significantly reduced fecal populations of wild-type *E. coli* (Anderson et al., 2000b). Based on these results, it has been suggested that cattle be treated with sodium chlorate immediately prior to transport for slaughter in order to reduce *E. coli* O157:H7 populations in the intestinal tract (Anderson et al., 2000a).

Chlorate treatment significantly reduced *E. coli* O157:H7, generic *E. coli*, and total coliform populations throughout the intestinal tract of experimentally infected cattle within 24 h (Figures 3a and b). It has been stated that much of the carcass *E. coli* O157:H7 contamination is associated with dried manure on the hide and hooves and is transferred to the carcass during hide removal (Grau, 1987); however, Elder et al. (2000) demonstrated that there was a direct correlation between fecal populations of *E. coli* O157:H7 and carcass contamination levels. Sodium chlorate treatment given 24 h prior to slaughter significantly reduced *E. coli* and *E. coli* O157:H7 populations in the feces (Figures 2a, 3a, and 3b) but did not affect *E. coli* O157:H7 populations on the hide in this study (data not shown). Counts of *E. coli* O157:H7 on the hides were extremely low due to animal cleanliness. However, it should be noted that these animals were housed in facilities atypical for the industry, and as a result the hides were relatively clean of feces or "tag." Collectively, these results indicate that sodium chlorate treatment immediately prior to slaughter could be an effective means of reducing *E. coli* O157:H7 carriage in the live animal, thus reducing carcass and subsequent food contamination.

Many intestinal bacteria do not have the enzyme nitrate reductase and are therefore insensitive to chlorate (Alaboudi, 1982). However, some important ruminal bacteria (e.g., *Selenomonas* and *Wolinella*) can reduce nitrate to nitrite and are sensitive to chlorate treatment. In our study, total culturable anaerobic bacterial populations were not affected by chlorate supplementation (Figure 4). Even though some prominent intestinal species are killed by chlorate addition, the gastrointestinal VFA profiles and pH were not significantly affected. *Escherichia coli* is able to become resistant to chlorate under in vitro conditions (Stewart, 1988); however, recent research with mixed fecal incubations from cattle indicated that chlorate-resistant *E. coli* O157:H7 mutants could not persist in competition against the chlorate-insensitive intestinal microbial population (Callaway et al., 2001).

Chlorate has been used as a herbicide and is toxic to rats ($LD_{50} > 1.2$ g/kg BW) (Material Safety Data Sheet; Sigma Chemical Co.). However, the toxicity of chlorate is actually quite low; in comparison the LD_{50} for NaCl in rats is approximately 3 g/kg BW, and the LD_{50} of acetylsalicylic acid is 200 mg/kg BW (Material Safety Data Sheet; Sigma). In the present study, cattle consumed approximately 0.4 g $NaClO_3$/kg BW. In previous studies, when chlorate was administered to rats, approximately 39% of chlorate was recovered in feces and urine within 24 h; however, chlorate did accumulate in the plasma (Fiume, 1995). Plasma chlorate is rapidly eliminated (α half-life of 6 h), and accumulation in carcass tissues was estimated to be less than 1 ng/g (Fiume, 1995). These data indicate that residue and toxicity

issues do not appear to preclude the use of chlorate as a feed additive to reduce *E. coli* O157:H7 populations in cattle immediately prior to harvest.

Implications

Escherichia coli O157:H7 is a significant threat to human health and the confidence of the American public in the safety of their food supply. Results of this study indicate that treating cattle with sodium chlorate reduced *E. coli*, total coliforms, and *E. coli* O157:H7 populations at a preharvest critical control point. Even though chlorate kills intestinal bacteria, those cells lacking nitrate reductase are unaffected by chlorate and in this study the gastrointestinal fermentation profile was not altered by chlorate supplementation. It appears that chlorate could be used to improve food safety, but further studies are needed to determine the most efficacious treatment regimen.

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